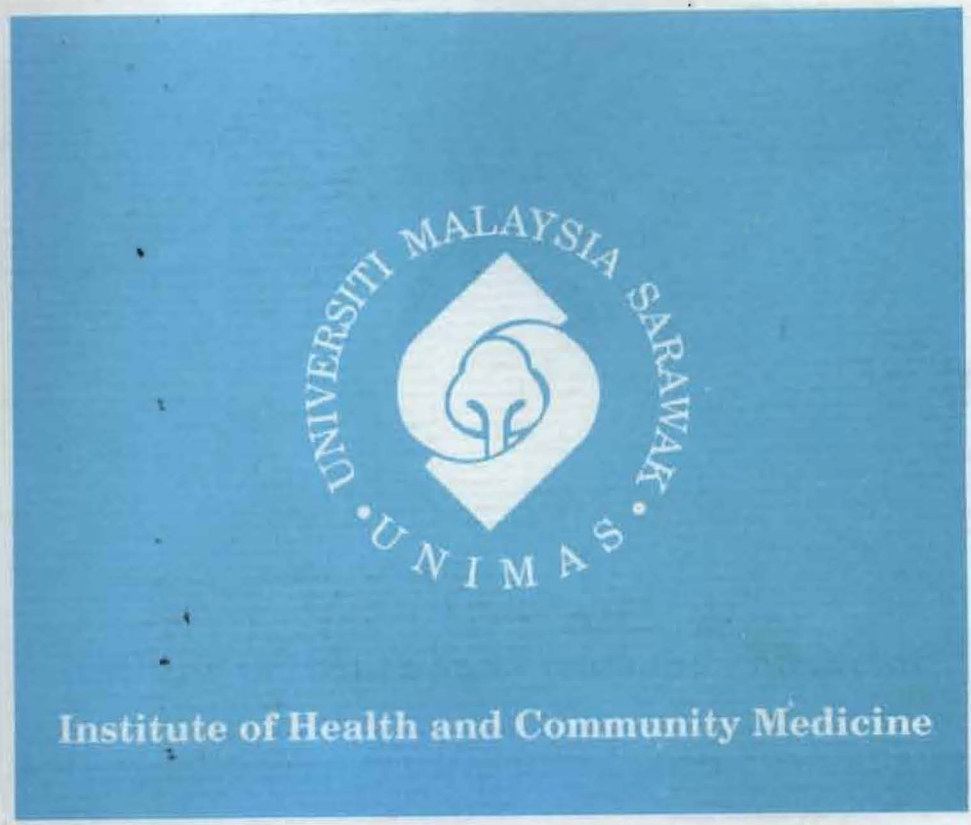

SENTINEL SURVEILLANCE OF HAND, FOOT AND MOUTH DISEASE IN SARAWAK, MALAYSIA, 1998-1999.

Edna Lyn Michael Gias

Kota Samarahan
2000

SENTINEL SURVEILLANCE OF HAND, FOOT, AND MOUTH DISEASE IN SARAWAK, MALAYSIA, 1998-1999.

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DEDICATION

To my family, for their constant prayer and faithful support.

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ABBREVIATIONS

3'NTR	3' nontranslated region
5'NTR	5' nontranslated region
AFP	acute flaccid paralysis
bp	base pair
CCA	conventional culture assay
CDC	Center for Disease Control and Prevemtion
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CPE	cytopathic effect
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DTP	diptheria-tetanus-pertussis
EV	enterovirus
HFMD	hand, foot and mouth disease
IFA	indirect immunofluorescent assay
IPV	inactivated poliovirus vaccine
LB	luria bertani
LBM	Lim Benyesh-Melnick
MMLV	moloney murine leukemia virus
NPEV	non-poliovirus enterovirus
OPV	oral poliovirus vaccine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RCA	rapid culture assay
RD	rhabdomyosarcoma
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
TAE	tris acetate EDTA
UHQ	utra high quality
VAPP	vaccine-associated paralytic poliomyelitis
WHO	World Health Organization

ABSTRACT

A sentinel surveillance study was carried out to monitor the incidence of hand, foot, and mou disease (HFMD) in Sarawak, Malaysia from March 1998 through August 1999. This study involved participation of seven sentinel clinics located in Kuching, Sibu, Miri and Sarikei. Children who w clinically diagnosed with HFMD were included in the study and the collected clinical specimens w inoculated onto the human rhabdomyosarcoma (RD) cells for the isolation of enteroviruses. The prese of enterovirus RNA in the culture fluids was confirmed with RT-PCR using pan-EV primer universal f enterovirus. The temporal distribution of HFMD shows an epidemiological pattern of HFMD cas where HFMD peaks in July, August and September 1998. The absence of these peaks in t corresponding period in 1999 suggests that occurrence of HFMD in Sarawak may not be seasonal. Mi HFMD peaks were also seen in April, November 1998 and March 1999. The clinical description HFMD in Sarawak did not differ from the classic descriptions of HFMD, and the characteristic of EV and non-EV71 HFMD were also shown to be similar.

The incidence specifically of EV71 HFMD was also studied. Culture fluids from pan-E positive children were subjected to RT-PCR using EV71 specific primer. The result shows that EV71 w detected during the period of April to September 1998. The monthly rate of EV71 detection vari between 5-20%. Overall, the rate of EV71 isolation during the surveillance period was only 6.7%. phylogenetic analysis based on the partial VP1 nucleotide sequences shows that the EV71 strains isolat during the surveillance period in 1998 belong to genogroup C. These Sarawak 1998 strains were al found to be closely related to three of the four Taiwan 1998 strains. One of the Taiwan strains belongs t genogroup B. Phylogenetic analysis of EV71 strains isolated during HFMD outbreak in Sarawak in 199 reveals that the strains were of B-type. The Sarawak 1997 EV71 strains isolated from geographically different towns in Sarawak were genetically similar and tightly clustered into an independent cluste within the genogroup B. The phylogenetic study of EV71 strains circulating in Sarawak and Taiwan al shows that there was no correlation between genetically different EV71 strains with the virulence of th viruses.

ABSTRAK

Pada Mac 1998 hingga Ogos 1999, satu pengawasan kawalan telah dijalankan untuk mengkaji insiden penyakit tangan, kaki dan mulut (HFMD) di Sarawak, Malaysia. Kajian ini telah melibatkan penyertaan 7 buah klinik terpilih di Kuching, Sibu, Miri dan Sarikei. Spesimen klinikal telah diambil daripada kanak-kanak yang disyaki mengidap penyakit HFMD dan dimasukkan ke dalam sel rhabdomyosarcoma untuk pengasingan enterovirus. Kehadiran semua RNA enterovirus di dalam kultur virus dikesan dengan primer 'pan-EV' dengan menggunakan kaedah RT-PCR.. Pemeriksaan terhadap pentaburan temporal telah mendapati bilangan kes HFMD meningkat secara mendadak di dalam bulan Julai, Ogos and September, 1998. Walaubagaimanapun, peningkatan di dalam bilangan kes tidak berlaku pada masa yang sama pada tahun 1999 dan berdasarkan kesimpulan ini, HFMD mungkin penyakit yang tidak bermusim di Sarawak. Peningkatan minor di dalam bilangan kes juga diperhatikan di dalam bulan April, November 1998 dan Mac 1999. Sifat-sifat klinikal HFMD di Sarawak didapati tidak berbeza daripada sifat-sifat klasik HFMD. Sifat-sifat klinikal HFMD yang disebabkan oleh EV71 jugak didapati tidak berbeza daripada sifat-sifat HFMD yang bukan disebabkan oleh EV71.

Insiden HFMD yang disebabkan oleh EV71 juga dikaji. Kajian ini telah dijalankan ke atas kultur virus yang telah ditunjukkan positif terhadap enterovirus. Kehadiran EV71 dikesan dengan primer yang spesifik kepada EV71 dengan menggunakan kaedah RT-PCR. Daripada keputusan yang diperolehi, EV71 hanya dikesan dalam jangkamasa April hingga September 1998 dan kadar pengesan EV71 setiap bulan berbeza dari 5% hingga 20%. Secara keseluruhan, kadar pengasingan EV71 sepanjang pengawasan penyakit HFMD hanya 6.7%. Analysis filogenetik yang berdasarkan jujukan nukleotida untuk sebahagian daripada gen VP1 menunjukkan bahawa EV71 yang telah diasingkan pada 1998 adalah ahli kepada 'genogroup C'. Kajian terhadap 3 daripada 4 jujukan nukleotida yang dimiliki oleh Taiwan 1998 EV71 mendapati bahawa virus tersebut juga berada di dalam 'genogroup C' manakala yang satu lagi berada di dalam 'genogroup B'. Analysis filogenetik untuk EV71 yang telah diasingkan semasa wabak HFMD di Sarawak pada 1997 mendapati bahawa virus ini dimiliki oleh 'genogroup B'. Sarawak 1997 EV71 yang diasingkan dari bandar-bandar yang berlainan di Sarawak adalah serupa dari segi genetik dan ia membentuk kluster yang unik di dalam 'genogroup B'. Analysis filogenetik untuk EV71 yang diperolehi di Sarawak dan Taiwan juga menunjukkan bahawa perhubungan di antara EV71 yang dimiliki oleh genogroup yang berlainan dengan kevirulenan tidak wujud.

CHAPTER 1. INTRODUCTION

1.1. PICORNAVIRUSES

The family of *Picornaviridae* comprises 9 genera: *Rhinovirus*, *Enterovirus*, *Aphthovirus*, *Cardiovirus*, *Hepatovirus*, (Miller, 1997) and 4 newly defined genera, *Teschovirus*, *Parechovirus*, *Erbovirus* and *Kobuvirus* (Pringle, 1999). The assignment of picornaviruses into specific genera has depended on the biological and physical properties but recently, the classification is mainly based on molecular properties of the viruses. The nucleotide sequences analysis of the member of *Enterovirus* genus has resulted in the reclassification of porcine enterovirus serotype 1 (PEV1), echovirus 22 (EV22) and echovirus 23 (EV23) into new genera. The phylogenetic analysis of the RNA-dependent RNA polymerase (Kaku *et al.*, 1999), P1 and 3D (Doherty *et al.*, 1999) sequences of PEV1 has shown that PEV-1 was distinct from the other members of *Enterovirus* genus and therefore was placed in a new genus *Teschovirus* and renamed porcine teschovirus. EV22 and EV23 have also been renamed parechovirus type 1 and type 2 respectively and assigned to a new genus *Parechovirus* (Mayo and Pringle, 1998). The previously unassigned Equine rhinovirus 2 was renamed Equine rhinitis B virus and has been placed in the genus *Erbovirus* (Pringle, 1999). Yamashita *et al.* (1998) have shown that a newly identified Aichi virus, an enteric virus associated with human gastroentritis, to be genetically distinct from all picornavirus genera and therefore this virus was assigned to new genus *Kobuvirus* (Pringle, 1999).

1.2. HUMAN ENTEROVIRUSES

1.2.1. Classification of human enteroviruses

One of the most important genus in family of *Picornaviridae* is *Enterovirus*. The member of the *Enterovirus* genus comprises viruses that infect human and animals. The classification of human enteroviruses into groups is based mainly on the virus pathogenicity in experimental animals and cytopathology in cell cultures. The enterovirus groups comprise polioviruses, coxsackieviruses group A and group B, echoviruses and higher numbered enteroviruses (Table 1). There are 66 immunological distinct human enterovirus serotypes (Miller, 1997), but presently EV22 and EV23 have been reclassified into a new genus of *Parechovirus* (Mayo and Pringle, 1998).

Table 1. The genus *Enterovirus* (Melnick, 1996a; Miller, 1997)

Group	Members
Polioviruses	PV1-PV3
Coxsackieviruses group A	CA1-CA22, CA24
Coxsackieviruses group B	CB1-CB6
Echoviruses	EV1-EV7, EV9, EV11-EV21, EV24-EV27, EV29-EV33
Enteroviruses	EV types 68-71

Polioviruses are the first member of enterovirus to be identified and they ab important causes of paralytic poliomyelitis. Further search for poliovirus from paralytic disea cl cases had led to the discovery of cosackieviruses, which are further divided into two differ groups based on their pathogenicity in newborn mice. Coxsackieviruses group A (CAV) ca lu a generalized myositis in the skeletal muscles that results in flaccid paralysis, wher coxsackieviruses type B (CBV), in addition to skeletal muscles, are able to affect wider ra of tissue including brain, spinal cord, liver, heart muscle and exocrine pancreas (Hyypiä a Stanway, 1993; Melnick, 1996a). The introduction of cell culture techniques has lead to t discovery of another enterovirus group – the echovirus (ECHO =enteric, cythopathoge human, orphan). Echoviruses were not associated with any diseases at the time of th discovery, as they did not cause characteristic pathogenicity in experimental anima However, it was later known to cause a wide range of clinical syndromes (Melnick, 1996a). more newly identified enterovirus shared similar properties with enterovirus of differe groups, the task of assigning them to groups become complicated. Therefore, the new identified enterovirus serotypes have been given numbers in the order of their identificatio for example enterovirus type 68 to 71.

Recent developments in molecular biology have enabled the analysis of th enterovirus nucleotide and amino acid sequences and such data are becoming more useful i the classification of enterovirus. The comparison of amino acid sequences in the P1 regio between representatives of picornavirus has shown that EV22 and EV23 are geneticall distinct from other enteroviruses and picornaviruses (Hyypiä *et al.*, 1997). The comparison o amino acid sequences in the VP1 and 2C regions between the representatives of picornaviruse have also shown the clustering of EV22 and EV23 into independent genetic cluster (Stanway e *al.*, 1994; Ghazi *et al.*, 1998). Apart from sharing some similar physical properties with othe enteroviruses, EV22 and EV23 have other properties atypical of enteroviruses. These includ the lack of VPO cleavage and myristoylation and the presence of a unique N-termina extension to VP3 and an arginine-glycine-aspartic acid (RGD) motif in VP1 (Stanway *et al.* 1994; Ghazi *et al.*, 1998). On the basis of these molecular and biological characteristics, EV2 and EV23 have been reclassified into a new genus of *Parechovirus* (Mayo and Pringle, 1998).

The phylogenetic analysis of several genomic regions has shown the genetic relationships among human enteroviruses. However in many cases, these relationships are no consistent with the classical grouping of enterovirus serotypes based on antibody neutralizatio tests. The comparison of nucleotide and deduced amino acid sequences in the capsid protei region (P1), partial sequences of VP4/2 and 3D regions resulted in the grouping of enteroviruses into four major phylogenetic clusters- A, B, C and D (Hyypiä *et al.*, 1997). Hyypiä *et al.* (1997) have suggested that the genotypic cluster C can be further divided into 2 subgroups based on the virus interaction with the host cell receptors to separate polioviruses from coxsackieviruses. The comparison of complete VP1 sequences of 57 human enterovirus strains has also produced 4 major phylogenetic clusters (Oberste *et al.*, 1999b), which is consistent with the published enterovirus phylogenetic trees based on the VP2 sequences (Hyypiä *et al.*, 1997). The enterovirus within each of the 3 major clusters (Table 2) is further segregated into distinct subgroups, with cluster A, B, and C have 3, 8 and 4 subgroups respectively (Oberste *et al.*, 1999b).

Table 2. Genetic classification of human enteroviruses based on complete VP1 gene nucleotide sequences (Oberste *et al.*, 1999b).

Cluster	Subgroup	Members
		CA2
	(i)	CA7, CA14, CA16 and EV71
A	(ii)	CA3,CA4,CA6,CA8 and CA10
	(iii)	CA5 and CA12
	(i)	EV3 and EV12
	(ii)	EV11and EV19
	(iii)	EV2 and EV15
	(iv)	EV13 and EV69
B	(v)	CB1-CB6
	(vi)	EV1, EV4 and EV8
	(vii)	EV6
	(viii)	EV21, EV25, EV29 and EV30
	unstable subgroup	CA9, EV5, EV7, EV9, EV14,EV16,EV17,EV18, EV20,EV24, EV26, EV27, EV31, EV32 and EV33
	(i)	CA1, CA19 and CA22
C	(ii)	CA21, CA24 and EV34
	(iii)	CA11and CA15
	(iv)	CA13, CA17, CA18, CA20, PV1, PV2 and PV3
D		EV70 and EV68

1.2.2. Physical and chemical properties

Enteroviruses are small, spherical and non-enveloped RNA viruses. A X-ray crystallography has shown that the icosadhedral capsid enclosing the RNA genome is roughly 5 nm thick and 30 nm in diameter (Rueckert, 1996). The buoyant density of enterovirus in CsCl is 1.34 g/ml and the molecular weight is 8.25 x 10^6 daltons (Zeichhardt, 1992). Enteroviruses are relatively stable viruses, shown by their resistance to disinfectants such as alcohol and lysol, and detergents such as ether and deoxycholate, but rapidly inactivated by ultraviolet light and free chlorine residual (Melnick, 1996a and 1996b). Enteroviruses are also thermolabile and they are destroyed rapidly at 50°C unless stabilized by $MgCl_2$ (Melnick, 1996a and 1996b). Another important property of enterovirus is their stability in acidic condition at pH ≤3 (Melnick, 1996a). This acid stability property enables the viruses to replicate in gastrointestinal tract, which is also their natural habitat.

1.2.3. Genome organization

The enterovirus genome consists of a single stranded positive sense RNA molecule approximately 7.5kb in length and a molecular weight of 2.6 x 10^6 daltons (Zeichhhardt, 199 The genome is organized into a 5'nontranslated region (5'NTR), a long polyprotein cod region, a 3'untranslated region (3'NTR) and a 3'polyA tail (Figure 1). Covalently attached the 5' end of the genome is a small basic viral protein called VPg protein, which plays a important role in the initiation of RNA replication (Rueckert, 1996). The 5' and 3'NTRs a highly conserved between different enterovirus types and they contain elements essential f efficient RNA replication and translation (Rohll *et al.*, 1995; Xiang *et al.*, 1995, Ruecke 1996, Mirmomeni *et al.*, 1997). The polyprotein-coding region can be divided into P1, P2 a P3 regions. The P1 region consists of genes that encode 4 viral structural proteins, VP1 to VP which make up the enterovirus capsid. The sequences of the P1 coding region are high variable because the viral capsid protein is constantly subjected to pressure caused by ho antibodies. The nucleotide sequences in P2 and P3 regions are highly conserved because th encode viral enzymes that are important in the virus life cycle. During the multiplication enterovirus in cell cytoplasm, the messenger active RNA is immediately translated into a lar polyprotein by using the host protein synthesis machinery (Rueckert, 1996). The existence this nascent polyprotein is transient as it is rapidly cleaved by viral-coded proteases into vir proteins. The replication of viral RNA is accomplished by viral coded RNA-dependent-RN polymerase.

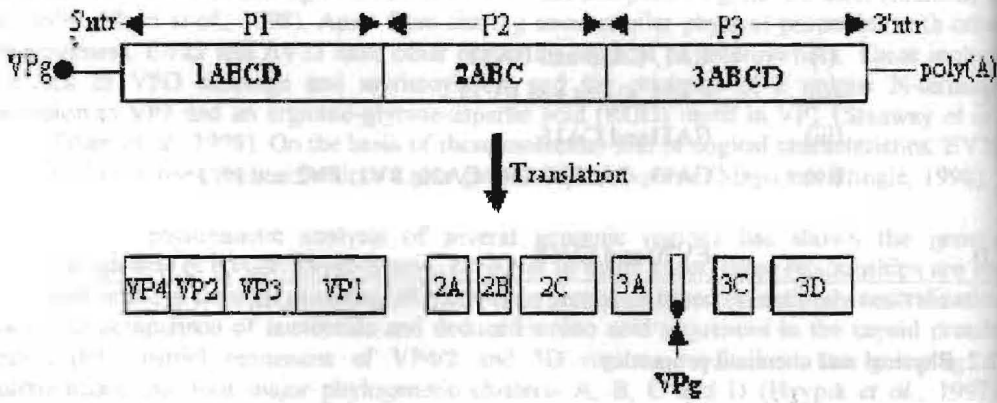

Figure 1. Structural organization of enterovirus genome (Melnick, 1996a). The genome is translated into a long polyprotein, which is then cleaved into individual peptides. The cleavag of polyprotein is performed by proteinase 2C and 3C or by a precursor form called 3CD.

.3. ENTEROVIRUS INFECTIONS

.3.1. Pathogenesis of enteroviruses

Enteroviruses gain entry into the human body mainly via the oral and respiratory routes. Once they are in the host body, they multiply in the gastrointestinal and respiratory tracts. In some cases, enterovirus may enter the blood stream. During this viremic phase they reach the target tissue such as the central nervous system (CNS), heart, muscles and skin, where further multiplication takes place and resulted in clinical diseases. In both asymptomatic and symptomatic infections, enterovirus can be recovered from the oropharynx and intestine. The viruses are generally shed for a longer period of time in the stool (Schnurr, 1992; Grist and Bell, 1996; Melnick, 1996a).

1.3.2. Clinical manifestation of enterovirus infections

Enterovirus infections can result in a wide spectrum of clinical syndromes. However, the most common forms of infection are asymptomatic or mild. Several specific clinical syndromes are associated with certain enterovirus serotypes, for example EV71 and CA16 are associated with hand, foot and mouth disease (HFMD) and coxsackieviruses group B are associated with myocarditis. However, there are instances whereby certain clinical syndromes are associated with more than one enterovirus serotypes, for example aseptic meningitis commonly caused by CBV2, CB5 and EV30 (Melnick, 1996a).

One of the most important clinical manifestations of enterovirus infection is paralytic poliomyelitis caused by polioviruses, but currently, this disease is effectively prevented by mass vaccination. Non-poliovirus enterovirus (NPEV) infections are becoming more important cause of morbidity and mortality. In a NPEV surveillance in the US (1993-1996), 3209 of NPEV isolations were reported, mainly from patients clinically diagnosed with aseptic meningitis, encephalitis, pneumonia, paralysis and carditis (CDC, 1997). Enterovirus is also the most important cause of aseptic meningitis in young infants and children, and among the serotypes commonly associated with aseptic meningitis are CB2, CB5, EV4, EV6, EV9, EV11, EV16 and 30 (Rorabaugh *et al.*, 1993; Melnick, 1996a). The epidemic of aseptic meningitis has also occurred in various parts of the world, and normally it is caused by a single predominant serotype, such as EV30, which responsible for outbreaks in Japan and Switzerland. (IASR, 1998b; Gorgievki-Hrisoho *et al.*, 1998). Other CNS diseases caused by enterovirus include encephalitis and poliomyelitis-like diseases, paralytic disease and acute flaccid paralysis and among the most important cause is EV71 (Alexander *et al.*, 1994). Myopericarditis, pleurodynia, upper respiratory illness and pneumonia are commonly associated with group B coxsackievirus infections (Melnick, 1996a). Herpangina are commonly caused by CA2, CA6, CA8 and CA10 while hand, foot, and mouth disease (HFMD) by CA16 and EV71 (Melnick, 1996a). Enterovirus infection is also common in neonates (Juhela, 1998) and tends to result in serious or even fatal diseases due to the immaturity of the immune system. Among the serious outcome of enterovirus infections in neonates are myocarditis with or without encephalitis, hepatitis, sepsis and pneumonia, and enterovirus serotypes frequently implicated are CB5, EV20 and EV11 (Galama, 1997, Head *et al.*, 1999). Severe enterovirus infections are also occurred in immunocompromised patients such as patients with aggammaglobunaemia and AIDS (Galama, 1997). Enterovirus has also been associated with chronic diseases such as insulin-dependent diabetes mellitus (Hiltunen *et al.*,

1997; Graves *et al.*, 1997), dilated cardiomyopathy (Conaldi *et al.*, 1997; Badorff *et al.*, 19 postviral fatigue syndrome and chronic cardiovascular disease (Melnick, 1996a).

1.4. LABORATORY DIAGNOSIS OF ENTEROVIRUS INFECTIONS

1.4.1. Virus isolation in cell culture

The diagnosis of enterovirus infections based on clinical ground alone is not reliab as the clinical syndromes may overlap with those caused by bacteria pathogens or oth viruses. Therefore, a laboratory diagnosis is necessary to confirm enterovirus infectio Currently, the diagnosis of enterovirus infections depends on the conventional method of virus isolation in tissue culture. The availability of different cell lines that are sensitive to enterovirus enables the isolation of many enterovirus serotypes, except for CA1, CA19 and CA22, whi can only be isolated in suckling mice (Gradlen *et al.*, 1989). Among the cell lines used isolation of enterovirus are the human rhabdomyosarcoma cells, HeLa cells, human fe diploid kidney cells, green monkey cells and primary monkey kidney cells (Gradlen *et a* 1989).

Although sensitive, the utility of tissue culture technique in enterovirus diagnosis is limited by several disadvantages. Virus isolation is time-consuming and normally several day are required before growth can be detected. Abzug *et al.*, (1995) has shown that after the inoculation of cerebrospinal fluid (CSF), serum, urine, throat and rectal specimens fro neonates onto the monkey kidney, Hep-2, Vero and human lung fibroblast cells, the mean tim to the earliest positive result for each patient was 4.2 days. Rotbart, (1989) has also reported mean of 6.3 days for the isolation of enterovirus from CSF specimens. The slow growth enterovirus in culture might be due to several factors. Among them is the low titer of virus particles or the presence of difficult isolates or mixture of viruses in the clinical specimens Several subpassages are also necessary before establishing a diagnosis of enterovirus infectio to rule out cytotoxic effect of some clinical specimens, which could be mistaken fo enterovirus cytopathic effect (CPE). The condition, type and quality of clinical specimens ar also important to ensure the virus remains viable. The insensitivity of cell line to certain enterovirus serotypes necessitates the use of multiple cell lines to improve the isolatio efficiency, a procedure which is expensive and labor intensive. Some enterovirus serotypes such as CA1, CA19 and CA22 require isolation in suckling mice and such technique is no suitable for use in the diagnostic laboratory. The tissue culture technique is also dependent on the access of laboratory with tissue culture facilities and requires high level of expertise. Other methods used for detection of enterovirus in clinical specimens include electron microscopy, immunofluorescence and enzyme-immunoassay (Schnurr, 1992; Gradlen *et al.*, 1989).

Although most enterovirus infections are self-limiting and require no specific therapy, rapid and accurate diagnostic test is required. This is because many enterovirus infections are often indistinguishable clinically from other treatable diseases caused by bacteria pathogens, and other viruses. The time-consuming procedure in tissue culture technique often results in unnecessary or longer hospitalization and unnecessary treatment with antibiotics or expensive antiviral drug such as acyclovir to treat herpes simplex infections. Enterovirus infections may also result in severe and potentially fatal diseases, especially in neonates and immunocompromised patients where immediate clinical decisions need to be made. Several diagnostic techniques have been developed to improve enterovirus diagnosis. Rotbart (1989 and 1991) has reported a rapid enterovirus diagnosis by using nucleic acid hybridization

chnique. This technique make use of the conserved sequences existed among enterovirus to velop broadly reactive probes such as nick-translated cDNA probes, single-stranded RNA robes and oligomeric DNA probes. Although rapid and sensitive, the clinical application of his hybridization technique is limited by low titer of virus in the certain clinical specimens. To vercome this, Rotbart (1989 and 1991) employed 'monolayer blot' technique and enzymatic mplification of a target region in the enterovirus genome to increase the number of target NA molecules available for hybridization with the probes. Bourlet *et al.*, (1998) have escribed a rapid culture assay (RCA) as an alternative to conventional culture assay (CCA) r detection of enterovirus in stool specimens. The RCA combined an immunoperoxidase test nd group specific anti-VP1monoclonal antibody to detect enterovirus CPE. The anti-VP1 nonoclonal antibody was specific for a highly conserved epitope in the VP1 of most nterovirus serotypes. This study has demonstrated that the sensitivity and specificity of RCA, ompared to CCA, were 77.9% and 98% respectively. Although rapid and more sensitive than CA, RCA is limited by the stool toxicity, which can reduce the sensitivity of the assay.

1.4.2. Serological diagnosis

Serological diagnosis plays a limited role in enterovirus diagnosis because of a large number of serotypes. Therefore, detection of type-specific antibody against enteroviruses is complicated by the lack of a single common antigen and occurrence of heterotypic antibody responses. Neutralization test is the most common test used to detect type-specific antibody. For serological diagnosis, a significant increase in antibody titer from acute and convalescent phase sera indicates enterovirus infection. Testing for the presence of type-specific antibody is made much easier when a characteristic clinical picture implicates certain serotypes such as group B coxsackieviruses which cause myocarditis, or an epidemic situation caused by one or few prevalent serotypes. The detection of enterovirus-specific IgM is valuable to determine whether the infection is recent. However, since the background level of IgM may be high the population, the results of IgM detection in serum must be interpreted with caution.

1.4.3. RT-PCR

The development of PCR technologies has greatly improved the diagnosis of enterovirus infections. Its simple procedure, rapidity, sensitivity and specificity have made PCR a more attractive and preferable method in enterovirus diagnosis than tissue culture and other methods discussed above. RT-PCR allows detection of enterovirus directly from the clinical specimens. The knowledge of complete nucleotide sequences of many enterovirus serotypes has made possible the design of broadly reactive or serotype/strain-specific PCR primers which can be used in the detection of enterovirus RNA in clinical specimens as well as typing of the enterovirus isolates (Romero and Rotbart, 1993 and 1994).

A number of studies that compared the performance of RT-PCR assay with viral culture have demonstrated that RT-PCR assay is useful for rapid and reliable diagnosis of enterovirus infections. Schlesinger *et al.* (1994) have reported that enterovirus was detected in CSF by RT-PCR from 11 of 12 infants with definite or probable aseptic meningitis and 6 of 13 infants with possible aseptic meningitis. The sensitivity of RT-PCR was further supported by RT-PCR positive result in 6 infants in whom the CSF viral cultures were negative. In the same study, Schlesinger *et al.* (1994) have also reported that the application of RT-PCR in the diagnosis of aseptic meningitis could have shorten hospitalization compared to viral culture. In

a study by Tanel *et al.* (1996), enterovirus was isolated from the CSF, throat and rectal specimens from 9 of 90 children with possible meningitis. Six children had enterovirus isolated from CSF, compared to 7 children who had positive CSF RT-PCR. In two children who had negative CSF RT-PCR, enterovirus was isolated from throat and rectal swabs. The sensitivity and specificity of RT-PCR were 77.8% and 100% respectively, compared to 66.7% by virus culture alone. Ahmed *et al.* (1997) have found that the sensitivity and specificity of CSF RT-PCR compared to viral culture for the diagnosis of enteroviral meningitis in infant were 91% and 94% respectively. In a similar comparison study, 13 (34%) and 25 (66%) of 38 CSF specimens collected during a summer outbreak of aseptic meningitis were positive for enterovirus by viral culture and PCR assay respectively (Yerly *et al.*, 1996). PCR assay was also shown to be more sensitive and accurate in diagnosing viral CNS infections, which include those caused by enterovirus (Chartterjee, 1999; DeBiasi and Tyler, 1999). In a study of 16 enterovirus-infected neonates, Abzug *et al.* (1995) have shown the combined sensitivity of serum and urine RT-PCR was 88% compared to that of viral culture, which was 56%. Chezzi (1996) has developed a RT-PCR assay using poliovirus-specific primer for rapid detection of polioviruses in infected tissue culture fluids and clinical specimens. So far the application of RT-nested PCR in enterovirus diagnosis has not widely used compared to single-step PCR. Jeffrey *et al.* (1997) have employed RT-nested PCR to detect enterovirus in CSF specimens from patients with viral infections of the CNS. Enteroviral RNA was also detected by nested PCR in all 13 throat swabs from patients with upper respiratory symptoms and acquired nosocomial infections and in 5 of 6 stool specimens from patients with acute flaccid paralysis (Kuan, 1997).

The recently developed commercialized enterovirus PCR test kit from Roche Molecular Systems, Basel, Switzerland (Yerly *et al.*, 1996) has standardized the procedure of RT-PCR assay in enterovirus diagnosis. The evaluation of this test kit with CSF specimens from meningitis patients have demonstrated that the kit was more sensitive than viral culture (Kessler *et al.*, 1997; Vliet *et al.*, 1998; Gorgievski-Hrisoho *et al.*, 1998; Pozo *et al.*, 1998; Hadziyannis *et al.*, 1999). The test kit is based on a one-step reverse transcription and PCR with the incorporation of uracil *N*-glycosylase to prevent carryover contamination. The use of biotinylated primers allows the detection of PCR product using a microwell colorimetric system (Yerly *et al.*, 1996). Rotbart (1997) has determined the reproducibility of the enterovirus PCR test with CSF, serum, urine, throat and rectal specimens from patients with enterovirus infections and found that the reproducibility of this test kit was 99%. Taggart *et al.* (1998) have reported that the sensitivity and reproducibility of the enterovirus PCR test was enhanced with incorporation of commercially available coprecipitant, a polymeric carrier used for nucleic acid precipitation, during RNA extraction.

In addition to enterovirus detection in clinical specimens, RT-PCR has also been found useful for other purposes. For example, in a study by Byington *et al.* (1999), RT-PCR was used for epidemiological investigation of the incidence of NPEV infections in febrile and afebrile infants. RT-PCR is also used to detect enterovirus RNA in myocardial tissue from patients with dilated cardiomyopathy (Archard *et al.*, 1998) and in formalin-fixed paraffin-embedded myocardial tissue from patients with acute myocarditis (Nicholson *et al.*, 1995). Behan *et al.*, (1996) have applied RT-PCR to identify the potential role of enterovirus in initiating autoimmune attack in inflammatory myopathy. A number of reports have also described the application of RT-PCR method for the detection of enterovirus in environmental samples (Lees *et al.*, 1994; Puig *et al.*, 1994; Reynolds *et al.*, 1996).

4.4. Typing of enterovirus

For the purpose of clinical diagnosis, the identification of individual enterovirus serotype is not normally carried out, as the traditional method of neutralization test takes a long time to benefit the patients. Although typing of enterovirus isolates is not crucial for clinical diagnosis, there are several situations whereby identification of enterovirus serotype is important. In a review, Muir *et al.*, (1998) has indicated that enterovirus typing is necessary for correct identification of poliovirus, study of enterovirus pathogenicity, identification of new enterovirus types, typing of enterovirus isolated from neonates and patients with chronic enterovirus infections and also for epidemiological purposes. The availability of intersecting pools such as the Lim Benyesh-Melnick (LBM) and the National Institute of Public Health and the Environment (RIVM) pool schemes has made easy the task of identifying most enterovirus serotypes (Melnick, 1996a). Although enterovirus typing by neutralization test is generally reliable, its application is limited by several disadvantages. Apart from the limited supply of antisera, time-consuming procedure, labor intensive and expensive, neutralization test is also unable to identify "untypeable" enterovirus. The "untypeable" enterovirus may be due to a new or previously unrecognized enterovirus serotypes, the presence of more than one enterovirus serotypes in the clinical specimens or formation of non-neutralizable aggregates. The procedure of neutralization also lacks standardization and the results obtained may vary in different laboratory (Loon *et al.*, 1999).

As a result of these limitations, a need has arisen to look for a more rapid and reliable method of typing. Rigonan *et al.*, (1998) have evaluated the use of indirect immunofluorescent assay (IFA) using commercially available monoclonal antibody for differentiation of polioviruses and NPEV. Although rapid, this method is less sensitive, and in addition to cross-reactivity of monoclonal antibody with other NPEV, the number of enterovirus types identified is also limited.

The application of RT-PCR and sequencing of the amplicon is becoming more useful for typing of enterovirus. However this typing method is only limited to viruses for which sequence information is available. Serotype-specific PCR primers are normally derived from the protein coding genes such as VP1 and VP2. Archard *et al.*, (1998) have employed RT-nested PCR using enterovirus group-specific primers to detect enterovirus in myocardium of patients with dilated cardiomyopathy, and this was followed by characterization of enterovirus isolates by sequencing of PCR product. Kilpatrick *et al.*, (1998) have used 3 sets of PCR primers derived from the VP1 gene to differentiate poliovirus serotypes based on the length of the PCR products. Arola *et al.*, (1996) have reported the application of competitive RT-PCR using primers derived from the 5'NTR and VP2 caspid protein-coding regions of the picornavirus genome. By using this primer, enteroviruses were differentiated from rhinoviruses based on the length of the PCR products, and at the same time enable genetic typing of enterovirus by sequence analysis of the PCR products. Although various regions of the enterovirus genome have been used for typing, nucleotide sequence analysis of the VP1 region provides a more useful information as this region contains important sites for serotype specificity. Comparison of the complete VP1 sequences of prototype human enterovirus strains has shown good correlation between VP1 sequences and serotype (Oberste *et al.*, 1999a). Oberste *et al.*, (1999b) have designed degenerate deoxyinosine containing PCR primers from 3' half of VP1 and the 5'end of 2A for typing of clinical enterovirus isolates, and sequence analysis of the 450bp amplicon has also shown that the nucleotide sequences of this region correlated 100% with EV serotype. Other methods of typing has also been reported, for example, Holland *et al.*, (1998) have developed a technique of viral protein fingerprinting for

differentiation and characterization of clinical enterovirus isolates based on the unique pro Afri
patterns of each serotype. RT-nested PCR followed by restriction fragment le emi
polymorhism (RFLP) analysis has also been used for typing of enterovirus directly from vacc
clinical specimens (Kuan, 1996). nsta
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1.5. CONTROL OF ENTEROVIRAL DISEASES ymp
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Currently there is no antiviral drug available for prevention and treatment of hu tic
enteroviral diseases. A new anti-picornavirus drug called pleconaril is still under research e cl
it has been shown to have inhibitory against most enterovirus strains in cell culture (Pevea cci
al., 1999). The only method to prevent enteroviral diseases is through public health acti nig
such practice of good hygiene, improved sanitation and standard of living and epidemiologi ere
surveillance of potentially life-threatening enteroviral diseases. Vaccination is one of the m
effective ways of preventing enterovirus infection, but the only vaccine available is
poliovirus.

The current poliovirus vaccines in use are live, attenuated oral poliovirus (OP
vaccine and inactivated poliovirus vaccine (IPV). These vaccines contain all 3 poliovi
serotypes. The OPV vaccine has been used more widely due to its easy administration, lo
cost and ability to induce intestinal immunity and long-lasting immunity (Modlin, 19
Melnick, 1996a). The OPV viruses are shed in the feces by vaccinees and spread to
unvaccinated contacts. Such transmission of OPV viruses is considered to be advantageo
because high vaccine coverage can be achieved especially in area with low vaccine acceptan
levels. However, the transmission of OPV viruses to unimmunized contacts is potentially ris
as they can revert to virulent strains and cause vaccine-associated poliomyelitis. This happen
in the USA, where 125 of the 133 confirmed cases of paralytic poliomyelitis were vaccin
associated paralytic poliomyelitis (VAPP) (CDC, 1999b). Unlike OPV, the eIPV contains n
living viruses and therefore it is recommended for immunodeficient persons as well a
unvaccinated adults (Modlin, 1996). IPV can be incorporated with a diptheria-tetanus-pertuss
(DTP) vaccine and since living viruses are absent, there is no risk of getting vaccine-associate
poliomyelitis. Despite these advantages, IPV is not widely used especially in developin
countries because of its expensive cost and inability to induce intestinal immunity effectivel
as OPV vaccines. The absence of secretory antibody allows wild type poliovirus to multiply i
the gastrointestinal tract of vaccinees which later are transmitted to unvaccinated contact
(Melnick, 1996a). Therefore, IPV fails to block the transmission of wild polioviruses by fecal-
oral route.

An alternative strategy of vaccination which involved a sequential administration o
IPV and OPV has been recommended to prevent VAPP among recipients and to reduce th
risk of OPV viruses transmission to immunocompromised individuals (Modlin, 1996). Wit
the implementation of this new vaccination schedule (CDC, 1999b) and the increase
capability to diagnosed non-polio enterovirus (NPEV) paralysis, the incidence of VAPP i
reported to be decreasing (Sepkowitz, 1997).

In 1988, the World Health Organization (WHO) aimed to eradicate poliomyeliti
globally by the year 2000 (WHO, 1988) by mass vaccination. As a result, the number o
poliomyelitis cases has greatly reduced. Presently wild polioviruses have been successfully
eradicated from the region of America by vaccination, and the effort to eradicate wild
poliovirus is still in progress in the endemic regions such as Europe, Asia and several countries

Africa (WHO, 1996). However, outbreaks of poliomyelitis commonly occur in these endemic regions. For example, in Angola in 1999, an outbreak of poliomyelitis has occurred in unvaccinated and partially vaccinated children and resulted in 39 deaths (CDC, 1999a). Constant monitoring of poliomyelitis cases is important in order to evaluate the progress towards global eradication of poliomyelitis. However the absence of poliomyelitis cases is not a reliable indicator of eradication as the most common infections by polioviruses are asymptomatic. Therefore it is very important that the evaluation of eradication is accompanied by virological monitoring. Virological monitorng is also important to exclude NPEV, in particular EV71, as they are able to cause paralytic disease and acute flaccid paralysis, which are clinically indistinguishable from poliomyelitis caused by poliovirus. To date, there is no vaccine available to prevent other enteroviral diseases since most of them are self-limiting and benign. However in view of the recent increase in EV71 outbreaks in several regions in Asia, there is a reason to develop an effective vaccine against EV71.

CHAPTER 2. STATEMENT OF PROBLEM

2.1. HAND, FOOT AND MOUTH DISEASE

Hand, foot, and mouth disease (HFMD) is a common acute viral illness that primarily affects young children and infants. HFMD has been reported to occur in outbreaks or epidemics in various parts of the world. The disease is usually mild and self-limiting. After an incubation period of 4 to 6 days following exposure of the virus to an individual, the patient will begin to have fever and sore throat. About 2 days after the appearance of initial symptoms, small aches superficial ulcers develop rapidly from vesicles on the gum, tongue, buccal mucosa and palate. A day or two after that, small red spots, which eventually turn into vesicles, appear on the palms of hands, soles of feet, buttocks, axillae or other areas. The skin lesions may be fewer in number. Fever and skin rash usually subside rapidly, but ulcers may last more than a week. The patient with HFMD is contagious when the first symptoms appear and continue to shed virus in the stool for up to several weeks even after the disappearance of the symptoms. (Grist and Bell, 1996; Melnick, 1996a) The virus is mainly spread by direct contact with respiratory discharges, weeping vesicles and feces of the infected people, and outbreaks may occur in overcrowding and conditions of poor hygiene.

Currently, there is no specific antiviral treatment for HFMD. Treatment is aimed at relieving fever and sore in the mouth. Practicing of preventive measures such as good personal hygiene is important to prevent the spread of the disease and potential outbreak from occurring. The affected individual normally develops specific immunity after the infection with a particular enterovirus serotype, but recurrent infection with different enterovirus serotypes may occur (Melnick, 1996a). Laboratory diagnosis of HFMD is rarely performed and is usually unnecessary due to the mild nature of the disease and the expense and length of time needed to perform the tests. The diagnosis is generally based upon the appearance of characteristic vesicles on hands and feet, ulcers in the mouth and mild fever.

The major causative agents of HFMD are CA16 and EV71. Several other enteroviruses such as CA4, A5, A9, A10, B2 and B5 are also known to cause HFMD (Grist and Bell, 1996; Melnick, 1996a;) but they are much less frequently associated with HFMD compared to CA16 and EV71. In an epidemiological surveillance in Japan (1982-1997), CA16 and EV71 were reported as the main agents of HFMD and in 1995 CA16 was responsible for a large-scale epidemic (IASR, 1998a). In the USA, CA16 was also reported as the agent most probably responsible for regional outbreaks of HFMD in 1989 (CDC, 1990). HFMD caused by CA16 is not associated with complications involving the CNS. However in rare cases infection with EV71 can cause meningitis, encephalitis or a poliomyelitis-like flaccid paralysis (Alexander *et al.*, 1994).

EV71 is first described in 1974 from patients in California who had severe neurological disease during an outbreak of CNS diseases between 1969 and 1973 (Melnick 1996a). Since its discovery, EV71 has been known to cause a variety of clinical syndromes among which are HFMD, aseptic meningitis, encephalitis, acute flaccid paralysis (AFP) and poliomyelitis-like diseases (Melnick, 1996a). A study by Silva *et al.* (1996) has associated EV71 with AFP-like poliomyelitis in Brazil after the detection of EV71 IgM in 20 of the 92 patients with AFP. Since asymptomatic infection is the most common form of infection, this

result must be interpreted carefully because the background level of IgM in general population may be high. In a surveillance for EV71 infection in children in Brazil from 1988 to 1990, EV71 has been shown as an etiological agent of acute neurological diseases such as AFP, Bell's palsy, acute cerebellar ataxia and Guillain-Barré syndrome (Takimoto *et al.*, 1998).

Outbreaks of EV71 infection have occurred in various parts of the world where severe neurological diseases including aseptic meningitis, encephalitis and poliomyelitis-like diseases have been reported. In an outbreak in Southern Sweden in 1973, 195 of 300 enterovirus strains isolated were EV71. The majority of patients from which EV71 was isolated had aseptic meningitis (Blomberg *et al.*, 1974). During a severe outbreak of CNS disease in Bulgaria in 1975 where poliomyelitis-like diseases, meningitis, meningoencephalitis and encephalomyocarditis were the main clinical syndromes manifested, 149 of 705 patients developed paralysis, and 44 of 149 patients who had paralysis died (Chumakov, *et al.*, 1979, Melnick, 1996a). A large-scale EV71 outbreak also occurred in Hungary in 1978 where the predominant clinical manifestation was aseptic meningitis. There were 724 encephalitis cases with clinical features such as acute cerebellar ataxia and poliomyelitis-like disease, and 45 of these cases were fatal (Nagy, *et al.*, 1982). The EV71 outbreaks with manifestation of CNS diseases have also been reported in Australia in 1972 (Kennett *et al.*, 1974) and in the USA in 1987 (Alexander *et al.*, 1994). In all of these outbreaks, a large number of cases with neurological manifestations were recorded and generally it was not linked with high mortality except for that in Bulgaria and Hungary. EV71 has also been associated with the outbreak of HFMD, for example in Japan in 1978 (Miwa *et al.*, 1980). The most recent outbreaks of EV71 HFMD have been in Sarawak in 1997 (Cardosa, *et al.*, 1999), Peninsular Malaysia in 1997 (Lum, *et al.*, 1998a and 1998b), Taiwan in 1998 (Ho, *et al.*, 1999; Huang *et al.*, 1999) and Perth, Western Australia in 1999 (McMinn, unpublished).

In Malaysia, HFMD had not been considered to be a public health problem since it is a mild disease that can be managed as outpatient. For this reason little attention has been given to the assessment of the incidence of the HFMD. However this perception changed after the occurrence of a cluster of pediatric deaths against a background of a large outbreak of EV71-associated HFMD in Sarawak in mid 1997 (Cardosa, *et al.*, 1999). The children who died presented with severe neurological disease and cardiac dysfunction, some with HFMD rashes (Cardosa *et al.*, 1999). Although the isolation of EV71 from some of these fatal cases has led to the suggestion that EV71 was the cause of death, the unusual clinical presentation together with the isolation of subgenus B adenovirus from fatal cases (Cardosa *et al.*, 1999) has raised a question of whether EV71 is the only etiological agent responsible for the deaths. During an outbreak of EV71 HFMD in Taiwan in 1998, similar clinical presentation in the fatal cases also occurred (Ho, *et al.*, 1999; Huang *et al.*, 1999).

2.2. OBJECTIVES OF PROJECT.

Prior to the 1997 outbreak, there is no published data on the EV71 infection in Sarawak, and therefore the epidemiological picture of EV71 HFMD is unknown. Although the etiology of death has never been satisfactorily proven due to EV71, the public is apprehensive about the real or perceived life-threatening nature of EV71 HFMD. Due to an increased concern among the public as well as the professional about EV71 HFMD, the Sarawak Health Department has implemented a sentinel clinic system for HFMD surveillance beginning in March 1998. This surveillance project involved the participation of seven sentinel clinics in